ELSEVIER

Aquatic Toxicology 76 (2006) 24–36

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors

Fang Zhang [a], Sigmund J. Degitz [b], Gary W. Holcombe [b], Patricia A. Kosian [b], Joseph Tietge [b], Nik Veldhoen [a], Caren C. Helbing [a,*]

[a] *Department of Biochemistry and Microbiology, P.O. Box 3055, Stn. CSC, University of Victoria, Victoria, BC V8W 3P6, Canada*
[b] *U.S. Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Blvd., Duluth, MN 55804, USA*

Received 7 April 2005; received in revised form 31 August 2005; accepted 7 September 2005

Abstract

Thyroid hormones (TH) are important in growth, development and the maintenance of proper cellular metabolism in vertebrates. Amphibian metamorphosis is completely dependent on TH and forms the basis of a screen for thyroid axis disrupting chemicals that currently relies on external morphological endpoints and changes in thyroid gland histology. The requirement for TH-dependent gene expression makes it possible to augment this screen through the addition of molecular endpoints. In order to do this, gene selection, choice of sampling time, tissue sensitivity, and their relationship to morphological change must all be considered. We exposed stage 54 *Xenopus laevis* tadpoles to a concentration series of the THs, thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3), and three known TH antagonists, methimazole, propylthiouracil (PTU), and perchlorate. The agonists significantly accelerated metamorphosis as defined by developmental stage attained after 14 days. In contrast, the TH antagonists significantly delayed metamorphosis at 14 days and caused an increase in thyroid gland size at day 8. We assessed the changes in steady-state mRNA levels of thyroid hormone receptor α- and β-isoforms and the basic transcription element binding (BTEB) protein by quantitative real-time polymerase chain reaction. Three tissues (brain, tail and hindlimb) were analyzed at 24, 48 and 96 h and we found that TH receptor, TRβ, and BTEB were the most sensitive gene transcripts for the TH agonists, whereas only TRα displayed significant changes upon antagonist exposure. We detected differences in tissue-specific responses between the two agonists. We matched the concentrations of T_3 and T_4 that elicited similar biological responses at 14 days and compared the induction of gene expression. At 96 h, the TRβ and BTEB expression response to T_3 and T_4 was similar in the tail. In contrast, T_3 elicited no concentration-dependent changes in TRβ and BTEB expression in the brain, whereas T_4 elevated their expression. The tail showed the highest correlation between TH concentration and morphological outcome whereas the brain was the most sensitive to antagonist treatment. Only methimazole and perchlorate showed significant changes in TRα gene expression in the brain whereas PTU did not suggesting differences in cellular mechanisms of action. The greatest effect on gene

* Corresponding author. Tel.: +1 250 721 6146; fax: +1 250 721 8855.
E-mail address: chelbing@uvic.ca (C.C. Helbing).

0166-445X/$ – see front matter
doi:10.1016/j.aquatox.2005.09.003

expression occurred within 48 h with many of the hormone-dependent changes disappearing by 96 h. This study accentuates the need to examine multiple tissues and provides critical information required for optimization of exposure regimens and endpoint assessments that focus on the detection of disruption in TH-regulatory systems.

Keywords: Gene expression; Metamorphosis; Tadpole; Thyroid hormone; Biomarker; Thyroid hormone receptor; Basic transcription element binding protein

1. Introduction

There is wide recognition that many chemicals in the environment may interfere with endocrine-regulated systems (Damstra et al., 2002) and there is a need to develop effective screens and biomarkers for their detection. One bioassay currently under development for detection of thyroid axis disruption is based on the use of the model amphibian species *Xenopus laevis*. This assay takes advantage of the dependence of amphibian metamorphosis on normal thyroid function and the use of whole animals to allow consideration of physiology, chemical biotransformation, and cellular context. Metamorphosis involves the transition of the aquatic tadpole to the terrestrial juvenile frog. The dramatic structural and functional changes of larval tissues during this developmental process are completely dependent upon thyroid hormones (THs) (Atkinson et al., 1996; Damjanovski et al., 2000). As in mammals, thyroxine (T_4) is the main secretory product of the amphibian thyroid gland (Buscaglia et al., 1985) and it is converted by deiodinase activity in peripheral tissues to the more active 3,5,3′-triiodothyronine (T_3) form (Becker et al., 1997; Huang et al., 1999; Kawahara et al., 1999). Metamorphosis has three distinct developmental phases: premetamorphosis, prometamorphosis, and metamorphic climax. During premetamorphosis, the tadpole is competent to respond to exogenous TH (which can induce a precocious metamorphosis) but is functionally athyroid. Prometamorphosis begins with maturation of the thyroid gland and low-level secretion of TH that initiates the first metamorphic changes such as limb growth. TH levels continue to rise and peak dramatically at metamorphic climax, which is characterized by the rapid, overt remodeling of the tadpole.

An amphibian metamorphosis assay based upon morphological endpoints is being developed for identifying disruptors of TH action using early prometamorphic *X. laevis* tadpoles (Degitz et al., 2005; Tietge et al., 2005). However, there is a need for the development of additional tools arising from the inability of standard toxicological approaches that focus on tissue and organismal effects to adequately discern molecular mechanisms of action. Gene expression monitoring is one approach that has the potential to serve as a reliable predictor of whole organism effects and provide crucial information for specific mechanisms of action of a particular chemical, class of chemicals or mixture of unrelated chemicals. Since changes in gene expression often precede overt morphological and physiological changes, development of molecular endpoints for incorporation into the existing assay could also reduce assay duration. TH-induced gene expression during anuran metamorphosis has been extensively studied and gene expression profiles associated with metamorphosis have been evaluated within different tissues of *X. laevis* (Buckbinder and Brown, 1992; Brown et al., 1996; Denver et al., 1997; Helbing et al., 2003). This wealth of information on tissue-specific gene expression programs of *X. laevis* during metamorphosis and the recent development of quantitative real-time polymerase chain reaction (QPCR) technology make it possible to examine endocrine disruptor (ED) effects on biological systems at the level of gene expression.

In order to do this, gene selection, choice of sampling time, tissue sensitivity, and the relationship of these factors to morphological change must all be considered. In the present study, we exposed Niewkoop and Faber (NF) (Nieuwkoop and Faber, 1956) stage 54 tadpoles to various concentrations of the thyroid hormones, 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4), and to the TH inhibitors, methimazole, propylthiouracil (PTU), and perchlorate. At 24, 48, and 96 h, we analyzed the expression of three candidate genes that are known to be TH-responsive: TRα, TRβ, and basic transcription element binding protein (BTEB). Gene

expression analysis was performed in the tail, brain and hindlimb to determine the relative sensitivities of these tissues to the gene expression endpoints. These tissues reflect diverse developmental TH-dependent fates, such as regression, remodeling, and growth, respectively. We compare their expression levels with morphometric endpoints derived at day 14. This work establishes fundamental assay criteria for the detection of TH disrupting chemicals and provides an important basis from which gene expression may be incorporated as an endpoint for the detection of disruption in TH-regulatory systems.

2. Materials and methods

2.1. Experimental animals

X. laevis tadpoles used in this study were obtained from an in-house culture where adults were injected with human chorionic gonadotropin to induce reproduction. The resultant embryos were treated with 2% cysteine (pH 8.1) for approximately 2 min to remove the jelly coat and rinsed thoroughly with Lake Superior water (LSW). Embryos were held at 21 °C for 4 days to allow for hatching, at which time they were divided into groups of 40 and maintained in LSW at 21 °C until the desired developmental stage was attained. Tadpoles were fed a blended mixture of TetraFin® (Tetra Sales, Blacksburg, VA, USA), *Spirulina* algae discs (The Wardley Corporation, Secaucus, NJ, USA), Silver Cup Trout Starter (Nelson & Sons Inc., Murray, UT, USA) along with live brine shrimp (Bio-Marine® Brand, Bio-Marine Inc., Hawthorne, CA, USA) twice/day on weekdays and once/day on the weekends regardless of treatment.

2.2. Water characteristics

Lake Superior water used for all tests was filtered through sand, a 5 μm filter, a 0.45 μm filter, sterilized with ultraviolet light and heated to the appropriate test tank temperature of 20.9 ± 0.2 °C ($n = 588$). Exposure tanks were immersed in a water bath system (water bath temperatures were continuously monitored) to maintain temperature uniformity between tanks. Dissolved oxygen (DO) was measured weekly during all tests using a DO meter (calibrated prior to use by the air saturation method). The range of DO measurements across all studies was 6.29–7.80 mg/l. All other water characteristics were measured using methods described by the American Public Health Association (APHA, 1992). The range of pH readings (conducted weekly on a minimum of 12 tanks during all tests) across all studies was 7.65–8.06. Hardness and alkalinity determinations were made on a minimum of three tanks (one control, two treatment tanks) once during each study. The range for total hardness across all studies was 47.0–47.5 mg/l $CaCO_3$. The range for all alkalinity measurements across all studies was 39.5–40.0 mg/l $CaCO_3$.

2.3. Chemicals

T_4, T_3, methimazole, 6-propylthiouracil (PTU) and perchlorate were obtained from Sigma Chemical Co. (St. Louis, MO). A stock solution of T_3 was prepared by dissolving 29 mg T_3 into 100 ml of 50 mM NaOH. A diluted stock solution of T_3 (234 nM) was prepared by addition of 10 ml T_3 stock to 19 l of LSW. T_4 stock solutions (5.16 μM) were prepared by dissolving 72.2 mg T_4 in 18 l of LSW. Stock solutions for methimazole (1250 mg/l) and perchlorate (492 mg/l) were made in a 19 l glass carboy by dissolving the chemical in Lake Superior water using a stir plate and a magnetic stir bar. Stock solutions for PTU (600 mg/l) were made in a 19 l glass carboy using a high speed top stirrer to dissolve the chemical in Lake Superior water that had been previously heated to 40 °C.

2.4. Exposure system

A computerized exposure system was used for all studies. This system, whose components are glass, stainless steel and Teflon, generated five duplicated exposure concentrations for each chemical with a dilution factor of 2 for T_4 and T_3, as well as duplicate controls. Exposure tanks were glass aquaria (22.5 cm × 14.0 cm × 16.5 cm deep) equipped with 13 cm standpipes, which resulted in an actual tank volume of 4.0 l. The flow rate to each tank was 25 ml/min. Fluorescent lamps provided a photoperiod of 12:12 h light:dark at an intensity that ranged from 61 to 139 lm at the water surface.

2.5. Animal exposures

2.5.1. Experiment 1 (T_4 and T_3)

Tadpoles were anaesthetized using 100 mg/l of tricaine methane sulfonate (MS-222) buffered with 200 mg/l of sodium bicarbonate, sorted by stage (Nieuwkoop and Faber, 1956), and allowed to recover from anesthesia in LSW. After recovery, 240 tadpoles (NF stage 54) were randomly placed into 12 tanks (20 tadpoles/tank) and exposed to five separate T_4 concentrations (10, 20.1, 40.3, 80.6, and 161.2 nM), and a LSW control. Remaining tadpoles (NF stages 52 and 53) were placed back in clean water until they reached stage 54 (3–4 days) when they were subsequently used in exposures with five different T_3 concentrations (0.48, 0.97, 1.92, 3.84, and 7.68 nM), and a LSW control. Each chemical exposure concentration was replicated twice along with the associated LSW control.

Once chemical exposure had begun, mortality observations were made daily and any dead tadpoles were removed. Water samples were taken at least three times throughout each test and measured for T_4 and T_3 concentrations. These measured concentrations were similar to the nominal concentrations (>97% nominal in all cases) and the effects data expressed as nominal concentration. At 24, 48 and 96 h, two organisms per exposure replicate (four organisms total per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* (Ambion Inc., Austin, TX, USA) for analysis of gene expression. On exposure day 14, all remaining organisms were euthanized in MS-222, weighed, and developmentally staged in a blind evaluation. No significant difference in the TH-induced rate of development between duplicate tanks was observed for either chemical exposure series ($p < 0.05$) and therefore, the combined results are presented. Other procedures or methods not specified followed those recommended by the American Society for Testing and Materials (ASTM, 2000).

2.5.2. Experiment 2 (methimazole, PTU and perchlorate)

Tadpoles were anaesthetized as in experiment 1, sorted by stage (Nieuwkoop and Faber, 1956), and allowed to recover from anesthesia in LSW. After recovery, tadpoles (NF stage 54) were randomly placed into 24 tanks (20 tadpoles/tank) and tadpoles were exposed (six tanks/chemical) to a single concentration of PTU (20 mg/l), methimazole (100 mg/l) or perchlorate (4 mg/l). These concentrations were selected based on the organism response that they have been shown to produce in previous studies (Degitz et al., 2005; Tietge et al., 2005).

Once chemical exposure had begun, mortality observations were made daily and any dead tadpoles were removed. Water samples were taken at least three times throughout each test and measured for PTU, methimazole, or perchlorate. The measured concentrations were similar to the nominal concentrations (>97% nominal in all cases) for all test chemicals (data not shown) and so the effects data presented are expressed as nominal concentration. At 24, 48 and 96 h, five tadpoles from two of the six tanks (10 tadpoles per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* (Ambion Inc., Austin, TX, USA) for analysis of gene expression. Additional tadpoles were sampled and processed for methods reported elsewhere (Degitz et al., 2005; Tietge et al., 2005). Other procedures or methods not specified followed those recommended by the American Society for Testing and Materials (ASTM, 2000).

2.6. Chemical analysis

Water samples collected from the T_3 and T_4 exposure chambers were immediately analyzed by high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) as described by Degitz et al. (2005). Water samples collected from PTU and methimazole exposure chambers were immediately analyzed by high-performance liquid chromatography as described by Degitz et al. (2005). Perchlorate was analyzed by ion chromatography as described by Tietge et al. (2005).

2.7. Isolation of RNA and cDNA synthesis

Brain, tail and hindlimbs were collected from each individual and total RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, Ont., Canada). Mechanical disruption of tissues utilized TRIzol reagent (300 μl for hindlimbs, 200 μl for brains, and 700 μl for tails), a 3 mm diameter tungsten–carbide bead, and safe-lock Eppendorf 1.5 ml microcentrifuge tubes in a Retsch

MM301 Mixer Mill (Fisher Scientific Ltd., Ottawa, ON) at 20 Hz for 6 min. Mixing chambers were rotated 180° halfway through the homogenization procedure. For the brain and hindlimb samples, 20 μg glycogen (Roche Diagnostics, Laval, PQ) was added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 20 μl (hindlimb and brain) or 40 μl (tail) diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at −70 °C.

Total RNA (1 μg from tail and hindlimb and 0.7 μg from brain) was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Baie D'urfe, Quebec, Canada) and cDNA was synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen Canada Inc., Burlington, Ont., Canada) as described by the manufacturer. The cDNA products were diluted 20-fold prior to PCR amplification.

2.8. Real-time quantitative polymerase chain reaction (QPCR)

The expression of selected gene transcripts was analyzed using a MX4000 real-time quantitative polymerase chain reaction system (Stratagene, La Jolla, CA, USA) as described previously (Crump et al., 2002) using gene specific primers (ViagenX Biotech Inc., Victoria, BC, Canada). A standard curve of each primer pair was generated using the appropriate quantified plasmid DNA containing the target gene sequence. Quadruplicate reactions were performed for each sample and data were averaged and normalized to the expression of the control gene encoding the ribosomal protein L8. The control gene expression was affected by TH antagonist treatment in the hindlimb at 24 h. In this case only, the expression data was normalized to the amount of input RNA. Gene expression data are presented as fold change relative to control animals within the same treatment period.

2.9. Statistics

Statistical analyses were conducted using SPSS software (SPSS, Chicago, IL, USA). Developmental stage was analyzed based on the distribution of stages at 14 days of exposure. The non-parametric Kruskal–Wallis one-way analysis of variance was conducted within hormone and tissue data sets. Where $p < 0.05$, pairwise comparisons were done using the Mann–Whitney *U*-test. The tank effect in every treatment concentration was not significant ($p > 0.05$) using the Mann–Whitney *U*-test, therefore, the data of two replicate tanks were combined for further statistical analyses. Paired examination of developmental stage attained between control and treated tadpoles was conducted using the Mann–Whitney *U*-test. Steady-state mRNA levels of the testing gene in a given exposure time and chemical concentration/treatment was compared with the respective controls using the Mann–Whitney *U*-test, with an α-adjusted significance level of $p \leq 0.02$. The correlation between the TH concentration-dependent gene expression in each tissue at a given exposure time and the developmental stage were analyzed by bivariate correlations. The averages of each parameter were used to determine Spearman's rho due to uneven sample sizes.

3. Results

3.1. Acceleration of development by THs

In order to assess tissue sensitivity, a concentration range of the agonists, T_3 and T_4, were chosen based upon reported endogenous values during natural metamorphosis (Leloup and Buscaglia, 1977; Krain and Denver, 2004). Within 14 days, NF stage 54 tadpoles develop to NF stage 60 (distribution range from 57 to 63; Table 1). To determine if exposure to the hormones affected metamorphosis, we assessed the developmental stages attained at 14 days of exposure. A significant shift ($p < 0.05$) in the distribution towards later developmental stages was observed even with the lowest concentration of T_3 (0.48 nM) examined. As T_3 exposure concentrations increased, this was accompanied by a concomitant increase in the number of animals at later developmental stages (Table 1). A shift in population distribution to later developmental stages (distribution range from 60 to 64) was also observed for the lowest concentration of T_4 (10 nM). This distribution is comparable to that obtained with exposure to 0.97 and 1.92 nM T_3 and reflects the known difference in biological activities of these THs (Table 1) (Frieden, 1968; White and Nicoll, 1981). With exposure to increasing T_4 concentrations, a further concentration-dependent shift to later developmental stages was also detected,

Table 1
Developmental stage distribution of *X. laevis* tadpoles exposed to TH for 14 days

Treatment	*n*	Mortality	NF stage								
			57	58	59	60	61	62	63	64	65
T_3 control	28	0	2	5	6	7	7	–	1	–	–
0.48 nM T_3	28	0	–	3	6	3	4	5	7	–	–
0.97 nM T_3	28	6	–	1	–	1	5	3	9	3	–
1.92 nM T_3	28	0	–	–	–	–	–	3	15	9	1
3.84 nM T_3	28	3	–	–	–	–	–	–	14	9	2
7.68 nM T_3	28	19	–	–	–	–	–	–	2	2	5
T_4 control	27	1	–	3	7	10	2	2	2	–	–
10 nM T_4	28	0	–	–	–	1	–	2	23	2	–
20.1 nM T_4	28	3	–	–	–	–	–	–	–	2	23
40.3 nM T_4	28	24	–	–	–	–	–	–	–	–	4
80.6 nM T_4	28	28	–	–	–	–	–	–	–	–	–
161.2 nM T_4	28	28	–	–	–	–	–	–	–	–	–

although this effect was restricted due to mortality. Mortality is frequently observed during precociously induced metamorphosis at higher hormone concentrations due to uncoordinated effects of gill resorption and lung development (Atkinson, 1981).

3.2. Gene expression response to exogenous TH

Significant TH-induced up-regulation of TRα ($p \leq 0.02$) was observed primarily in the tail and the magnitude of this induction remained within two-fold (Fig. 1). There was a progressive increase in the steady-state levels of TRα mRNA with increasing exogenous T_3 concentration at 24 and 48 h in the tail. At 96 h, a significant increase of TRα mRNA expression was detected only with the highest T_3 concentration relative to the control animals ($p \leq 0.02$). Up to a two-fold elevation in steady-state levels of TRα mRNA was observed at most T_4 concentrations for all treatment periods in the tail ($p \leq 0.02$). The brain showed no significant difference at any time point for T_3 or T_4 (Fig. 1). The hindlimb also showed no significant differences for T_3 or T_4 treatments except that a significant decrease was observed at the 96 h time point at concentrations above 10 nM T_4 (Fig. 1).

The temporal response in TRβ expression to T_3 exposure varied among tissues (Fig. 2). A progressive increase in steady-state levels of TRβ mRNA with increasing T_3 concentration was observed in the tail at all time points and significant fold-induction was evident at the lowest T_3 concentration at 24 and 48 h ($p \leq 0.02$). At 96 h, however, the magnitude of induction was reduced. Neither the brain nor the hindlimb showed a significant increase in TRβ levels at any time with the lowest T_3 concentration. At the higher concentrations, significant increases were detected beginning at 0.97 nM, except in the brain at 24 h, where a significant increase was detectable with at least 1.92 nM T_3. By 96 h, the levels of TRβ mRNA in both brain and hindlimb were indistinguishable from the control. The greatest increase in TRβ mRNA expression detected was approximately six-fold in tail and four-fold in brain and hindlimb.

The overall response in all tissues to T_4 exposure was similar to that observed for T_3 with some notable exceptions. TRβ transcript levels displayed a concentration-related increase in expression in the tail at 24 and 48 h which was maintained at 96 h (Fig. 2). In contrast to the lack of induction of TRβ levels by T_3 in the brain at 96 h, treatment with T_4 at this time point resulted in a modest maintenance of the induction response observed at 24 and 48 h (Fig. 2). In the hindlimb, the higher T_4 concentrations elicited a significant elevation in TRβ mRNA levels at 24 h ($p \leq 0.02$). However, this response was rapidly diminished to non-significance by 96 h.

In general, the TH-dependent induction of BTEB mRNA expression was strikingly similar to that of TRβ mRNA ($p \leq 0.02$; Fig. 3). However, BTEB transcript levels in the tail increased up to 14- and 35-fold for T_3 and T_4, respectively. For both hormones, BTEB mRNA levels reached a maximum five-fold in the brain

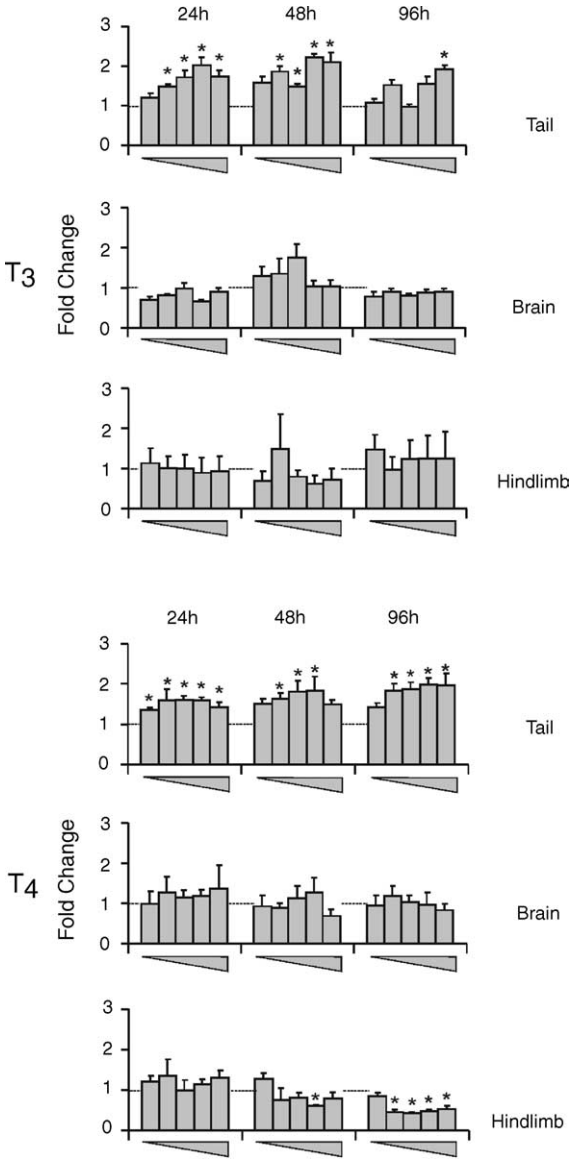

Fig. 1. TH-dependent changes in gene expression for TRα in *X. laevis* tadpoles. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to different concentrations of T_3 or T_4 at 24, 48, and 96 h of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Grey triangles represent the increasing two-fold increments in nominal exposure concentrations for T_3 (0.48, 0.97, 1.92, 3.84, and 7.68 nM) or T_4 (10, 20.1, 40.3, 80.6, and 161.2 nM). The dotted lines show mRNA abundance equal to the control steady-state levels. Error bars represent the S.E.M. Statistically significant differences (α-adjusted, $p \leq 0.02$) between treatment vs. control are denoted by an asterisk.

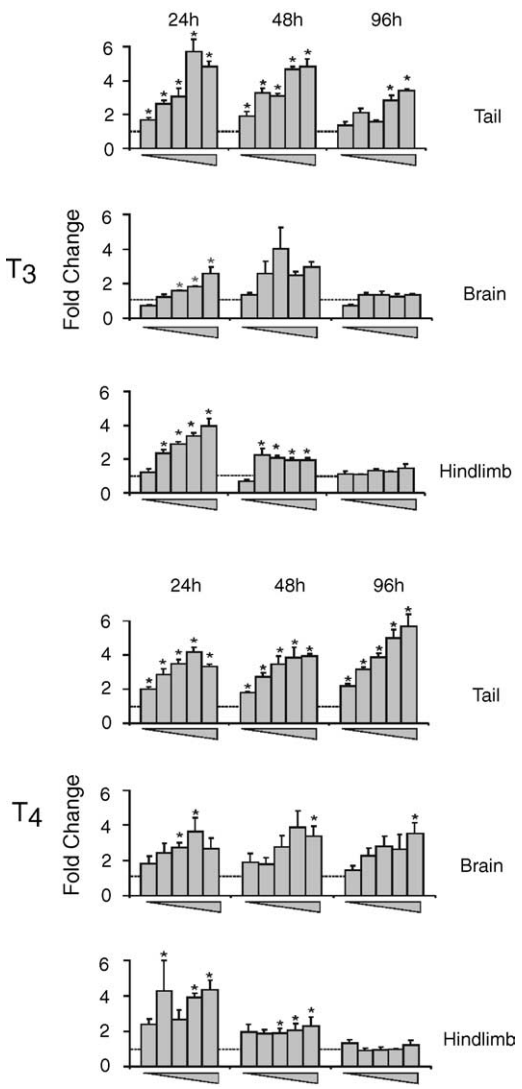

Fig. 2. TH-dependent changes in gene expression for TRβ in *X. laevis* tadpoles. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to different concentrations of T_3 or T_4 at 24, 48, and 96 h of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Grey triangles represent the increasing two-fold increments in nominal exposure concentrations for T_3 (0.48, 0.97, 1.92, 3.84, and 7.68 nM) or T_4 (10, 20.1, 40.3, 80.6, and 161.2 nM). The dotted lines show mRNA abundance equal to the control steady-state levels. Error bars represent the S.E.M. Statistically significant differences (α-adjusted, $p \leq 0.02$) between treatment vs. control are denoted by an asterisk.

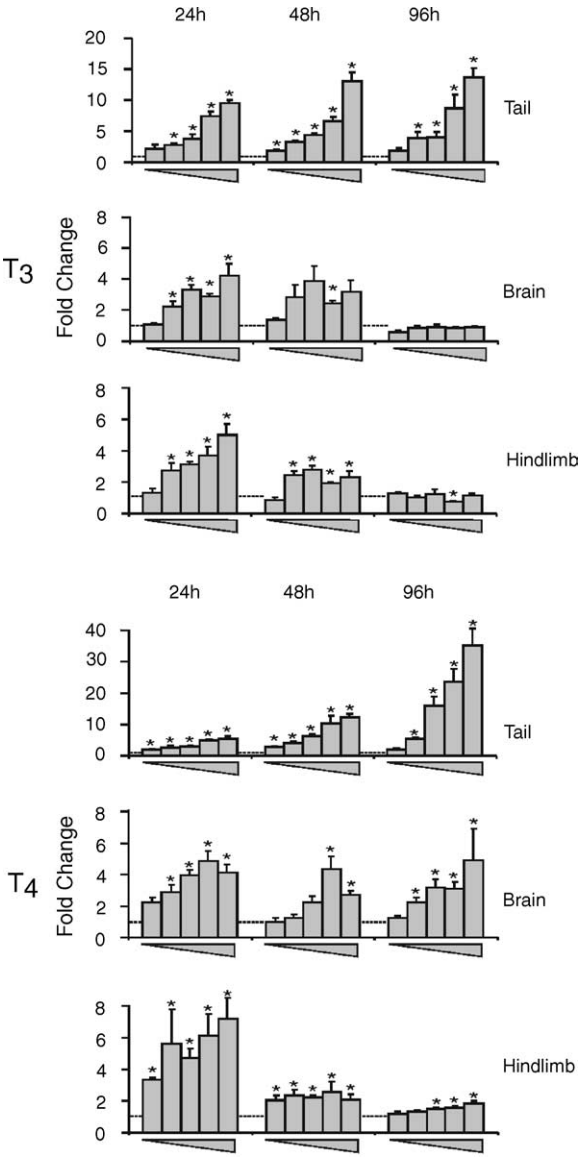

Fig. 3. TH-dependent changes in gene expression for BTEB in *X. laevis* tadpoles. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to different concentrations of T_3 or T_4 at 24, 48, and 96 h of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Grey triangles represent the increasing two-fold increments in nominal exposure concentrations for T_3 (0.48, 0.97, 1.92, 3.84, and 7.68 nM) or T_4 (10, 20.1, 40.3, 80.6, and 161.2 nM). The dotted lines show mRNA abundance equal to the control steady-state levels. Error bars represent the S.E.M. Statistically significant differences (α-adjusted, $p \leq 0.02$) between treatment vs. control are denoted by an asterisk.

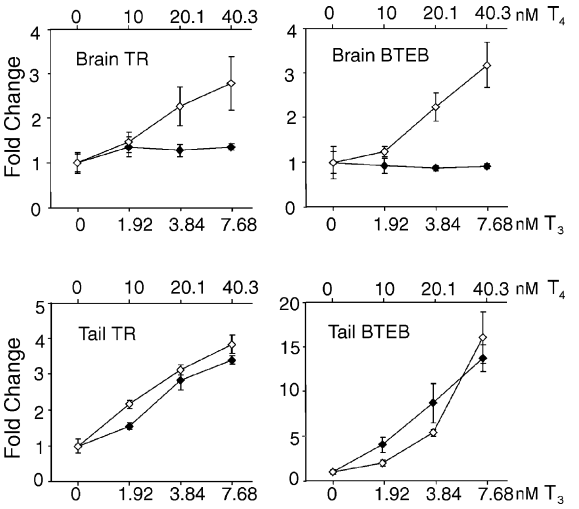

Fig. 4. Comparison of TRβ and BTEB expression in the brain and tail in response to T_3 and T_4 at 96 h. The response curves of each gene are plotted based upon the known differences in biological activities of T_3 and T_4 (Frieden, 1968; White and Nicoll, 1981) and our own morphological observations (Table 1). Data from the T_3 (closed diamond) and T_4 (open diamond) exposures are presented as fold change relative to matched time point controls.

and seven-fold in the hindlimb. A clear concentration-dependent pattern of induction was observed in the tail for both hormones.

In our analyses, we observed that the relative responses to T_3 and T_4 differed in the tail and brain. We matched the concentrations of T_3 and T_4 that elicited similar biological responses at 14 days and compared the induction of gene expression. At 96 h, the TRβ and BTEB expression response in the tail to T_3 exhibited almost identical patterns compared to their response to T_4 (Fig. 4). In contrast, T_3 elicited no concentration-dependent changes in TRβ and BTEB expression in the brain, whereas T_4 elevated their expression.

3.3. Gene expression response to TH inhibitors

We have previously shown that methimazole, PTU and perchlorate significantly inhibit the development of *X. laevis* tadpoles (Degitz et al., 2005; Tietge et al., 2005). In order to determine the potential effect that these inhibitors have on the expression of the three test genes, we separately exposed NF stage 54 tadpoles to 100 mg/l methimazole, 20 mg/l PTU, or 4 mg/l perchlorate. QPCR analyses of TRα gene expression

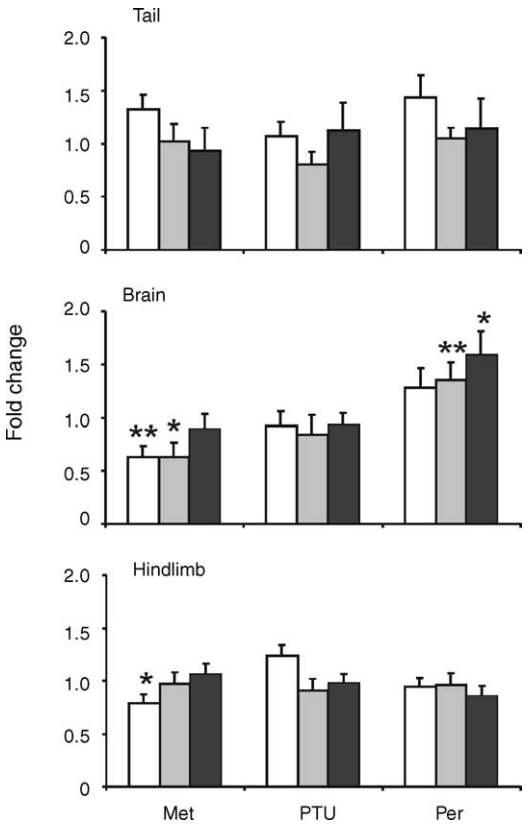

Fig. 5. Changes in gene expression for TRα in *X. laevis* tadpoles exposed to TH antagonists. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to 100 mg/l methimazole (Met), 20 mg/l PTU or 4 mg/l perchlorate (Per) at 24 h (white bars), 48 h (grey bars), and 96 h (black bars) of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Error bars represent the S.E.M. Statistically significant differences between treatment vs. control are denoted by $^{*}p \leq 0.02$ and $^{**}p < 0.001$.

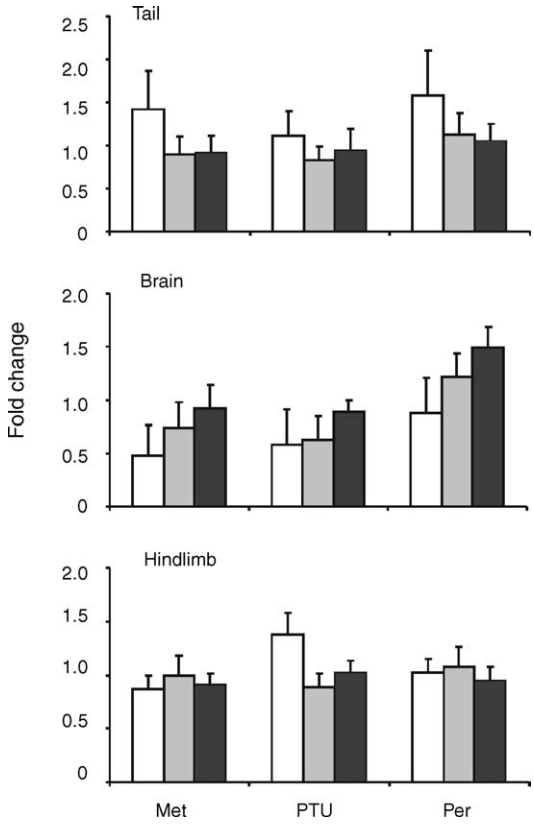

Fig. 6. Changes in gene expression for TRβ in *X. laevis* tadpoles exposed to TH antagonists. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to 100 mg/l methimazole (Met), 20 mg/l PTU, or 4 mg/l perchlorate (Per) at 24 h (white bars), 48 h (grey bars), and 96 h (black bars) of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Error bars represent the S.E.M. No statistically significant changes were observed in any tissue at any time point.

showed a significant decrease in the brain at 24 h ($p < 0.001$) and 48 h ($p \leq 0.02$) with methimazole treatment and a significant increase at 48 h ($p < 0.001$) and 96 h ($p \leq 0.02$) upon perchlorate treatment (Fig. 5). There was no significant effect of the PTU treatment at any time. Although the hindlimb showed a significant ($p \leq 0.02$) decrease upon methimazole treatment at 24 h, all other treatments and time points showed no significant change. No significant change was observed in the tail at any time or treatment.

The expression levels of both TRβ and BTEB were unchanged upon inhibitor treatment at all time points and in all tissues tested (Figs. 6 and 7).

4. Discussion

The development of bioassays that measure changes in gene expression show great promise in the detection of thyroid active agents and their disruptive effects on development and homeostasis. Of particular interest is the examination of chemicals that may possess endocrine disruptive activity and alter hormone-regulated gene expression programs. In the amphibian, postembryonic development is dependent upon the synthesis and activity of TH. Changes in TH-dependent gene expression can be detected by 48 h in most tissues following exposure to exogenous

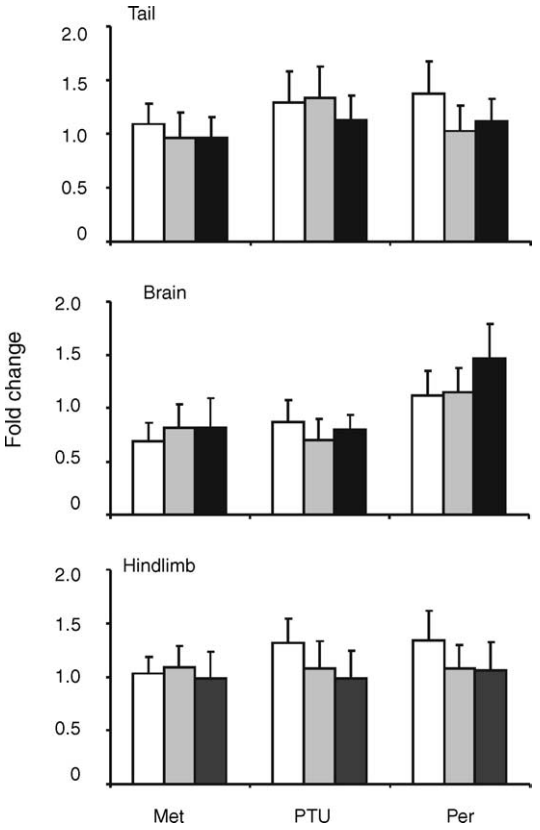

Fig. 7. Changes in gene expression for BTEB in *X. laevis* tadpoles exposed to TH antagonists. QPCR analysis was performed on mRNA isolated from tail, brain and hindlimb tissues exposed to 100 mg/l methimazole (Met), 20 mg/l PTU, or 4 mg/l perchlorate (Per) at 24 h (white bars), 48 h (grey bars), and 96 h (black bars) of treatment. Gene expression data are presented as fold change relative to control animals within the same treatment period. Error bars represent the S.E.M. No statistically significant changes were observed in any tissue at any time point.

TH (Buckbinder and Brown, 1992; Wang and Brown, 1993; Denver et al., 1997; Helbing et al., 2003). The establishment of molecular endpoints for incorporation into the metamorphosis assay must address a number of crucial experimental factors that play important roles in the interpretation of exposure data and the overall predictive ability of the assay (De Vito et al., 1999). These factors include developmental stage of the test animal population, selection of responsive gene expression biomarkers, choice of tissue sampled for gene expression assessment, the nature and concentration of test chemicals under investigation, the duration of exposure to the test chemicals, and the relationship between gene expression modulation and resultant biological outcome. In the present study, we examined T_3 and T_4 as representative TH agonists and methimazole, PTU and perchlorate as representative TH antagonists to establish the utility of select genes as molecular markers and to establish the timing of sample collection. The change in known TH-responsive gene transcript levels was examined relative to the rate of metamorphosis and, in the case of antagonists, thyroid histology, in order to form the basis of a predictive gene expression bioassay to identify agents that disrupt normal development.

Metamorphosis of the tadpole requires hormone-dependent initiation of tissue-specific gene expression programs that function to alter the proteome and ultimately tissue fate. Among the three candidate biomarkers examined in this study, both TRβ and BTEB mRNAs showed a strong relationship with T_3/T_4 exposure within all tissues examined, whereas TRα transcript levels did not. Within the confines of the experimental design, we were able to detect significant changes in some of the measured gene transcript levels even at the lowest concentrations examined. It would be important to perform exposures on lower concentrations of THs than those presently used to determine the limit of sensitivity of the QPCR-based assay.

Many of the TH-dependent gene expression studies to date have employed premetamorphic tadpoles and have suggested that the tail is relatively insensitive to low concentrations of TH during precocious metamorphosis. For example, induction of BTEB expression was detected in the brain of premetamorphic (NF stage 52) animals at 50 nM T_3 but a detectable up-regulation in tail tissue required a four-fold higher concentration of T_3 (Hoopfer et al., 2002). The present study, using QPCR in combination with NF stage 54 tadpoles in a flow-through exposure system, was able to detect significant induction of BTEB in tail tissue following administration of 0.48 nM T_3 (Fig. 3). In contrast, the hindlimb, which is generally regarded as a highly sensitive TH-responsive organ, did not show a high degree of sensitivity with the QPCR-based assay. Since early prometamorphic tadpoles have low-levels of endogenous THs, it is possible that these levels were sufficient to cause an increase in basal levels of test transcript levels in the hindlimb that would mask any further TH

Table 2
Spearman's rho correlation between TH-dependent gene expression and developmental stage achieved at day 14

Gene	Time (h)	T_3			T_4[a]		
		Tail	Brain	Hindlimb	Tail	Brain	Hindlimb
TRα	24	0.943*	−0.314	−0.771	1.000*	0.600	−0.200
	48	0.771	0.143	−0.429	1.000*	0.200	−0.400
	96	0.657	−0.200	0.257	1.000*	0.600	−1.000*
TRβ	24	0.943*	0.943*	1.000*	1.000*	1.000*	0.800
	48	0.943*	0.714	0.429	1.000*	0.800	0.400
	96	0.943*	0.429	0.886*	1.000*	1.000*	−0.600
BTEB	24	1.000*	0.943*	1.000*	1.000*	1.000*	0.800
	48	1.000*	0.714	0.486	1.000*	0.800	0.800
	96	1.000*	−0.143	0.029	1.000*	1.000*	1.000*

[a] Does not include data from 80.6 and 161.2 nM due to 100% mortality by day 14.
* Statistically significant $p < 0.05$.

exposure. Indeed, we did detect a significant increase in TRβ mRNA levels in control animals over the 96 h time period (data not shown).

In order for a gene expression endpoint to be useful, it must be able to predict a deleterious effect such as accelerated metamorphosis. We therefore determined whether temporal changes in TH-dependent gene expression that occur within 96 h for a given tissue correlate well with morphological outcomes that were measured 10 days later. We performed bivariate correlation analysis on the expression levels of each of the target gene transcripts compared to the developmental stage achieved at day 14 (Table 2). Although TRα mRNA levels correlated strongly with accelerated metamorphosis at some time points in the tail and hindlimb, it was not a good indicator in the context of T_3 exposure, nor was there a relationship detected in the brain at any time point (Table 2). In contrast, both TRβ and BTEB mRNA levels were strongly correlated with accelerated metamorphosis in the brain and tail at 24 h regardless of the TH agonist used (Table 2). This strong correlation was maintained in the tail for these two transcripts for 48 and 96 h suggesting that assessment of tail tissue allows for a wider time span (24–96 h) in which to observe significant hormone-dependent changes in TRβ and BTEB mRNA expression. At no time point tested in the hindlimb did both T_3 and T_4 treatments show a strong correlation between any transcript and acceleration of metamorphosis. Therefore, the brain and hindlimb display limitations in the magnitude and length of induction and thus are restricted in their applicability for use with these gene expression endpoints in identifying TH agonists.

Morphological changes that occur in the tadpole during metamorphosis are extensive and involve most tissues. Not all tissues appear to respond to equivalent concentrations of TH; limb development and remodeling of the brain occur early while regression of the tadpole tail is initiated late in the metamorphic process (Shi, 2000), and some require continuous exposure to a certain concentration of TH while others require only brief exposure (Atkinson, 1981). How specific tissues respond to specific TH agonists may also vary. We detected differences in tissue-specific responses between the two agonists (Fig. 4). This may be due to differences in TH import and export and/or the tissue-specific balance of different deiodinase activities. Alternatively, the observed difference between the two THs could be due to certain unique T_4 functions independent of its conversion to T_3. Whatever the cause(s), this observation accentuates the need to examine multiple target tissues for the assessment of gene expression biomarkers.

This finding is further extended in the examination of TH antagonists. We chose the three antagonists to represent different modes of action. Methimazole and PTU are goitrogens that inhibits TH production by blocking the formation of active iodide via the peroxidase system (Cooper et al., 1984) and perchlorate ions are competitive inhibitors of iodide uptake (Wolff, 1998). We chose concentrations that we previously showed caused an increase in thyroid gland

size at day 8 and significantly delayed metamorphosis at 14 days with no mortality (Degitz et al., 2005). All three antagonists have been used to therapeutically treat hyperthyroidism, but perchlorate is also a persistent environmental contaminant (Soldin et al., 2001) with environmental exposures in groundwater ranging from 200 μg/l to 3700 mg/l in highly contaminated sites (Urbansky, 1998).

A significant change in TRα gene expression was detected only in the brain with little or no effect on the tail or hindlimb. Methimazole treatment resulted in a down-regulation and perchlorate caused an up-regulation, whereas no effect was seen for PTU (Fig. 5). The gene transcripts that were strongly correlated with TH agonist function were not affected by any TH antagonist suggesting that the initial mechanisms of action at the gene expression level are fundamentally different. There is no precedent for a selective change of TRα transcript levels over this short time frame in these tissues, but the alteration seen may reflect the acute cellular response to inhibitor exposure. Although TRα transcript levels present an intriguing possible biomarker for methimazole and perchlorate exposure, the lack of response to PTU suggests that more detailed analyses are required to determine if TRα expression levels are indeed affected by this chemical, perhaps with different kinetics. For example, the effects of methimazole are not detected by 96 h (Fig. 5). It is possible that a transient down-regulation has occurred prior to the 24 h time point for PTU which was thus missed.

The data presented in this manuscript demonstrate the importance of determining the appropriate sampling time point and tissue. By focusing on three candidate biomarker gene transcripts at a 48 h exposure time, TH agonists were best identified in the tail with up-regulation of all three genes tested. Two TH antagonists were characterized by gene expression analyses in the brain as no change in TRβ and BTEB mRNA levels and a decrease (for methimazole) or increase (for perchlorate) in TRα mRNA levels. Further identification of additional candidate gene biomarkers would enhance the confidence in the designations of chemical treatments. The development of a metamorphosis-based assay that includes short-term gene expression endpoints and employs the highly sensitive QPCR methodology shows great promise as an effective ED screening tool.

Acknowledgements

We thank Jeff Ricketson and Rachel Skirrow for technical assistance. This work was funded by an EPA cooperative agreement and in part by the EJLB Foundation to CCH. CCH is a Michael Smith Foundation for Health Research scholar and is recipient of a NSERC University Faculty Award and an early career award for applied ecological research from the Society of Environmental Toxicology and Chemistry. This work was supported in part with funding provided by the American Chemistry Council through a Cooperative Research and Development Agreement (CRADA) with the EPA's Mid Continent Ecology Division. The information in this document has been funded in part by the U.S. Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References

APHA, 1992. Standard Methods for the Examination of Water and Wastewater. Washington, DC. American Public Health Association, American Water Works Association, Water Environment Foundation.

ASTM, 2000. Standard guide for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians. West Conshohocken, PA.

Atkinson, B.G., 1981. Biological basis of tissue regression and synthesis. In: Gilbert, L.I., Frieden, E. (Eds.), Metamorphosis: A Problem in Developmental Biology. Plenum Press, New York, pp. 397–444.

Atkinson, B.G., Helbing, C., Chen, Y., 1996. Reprogramming of gene expression during amphibian metamorphosis. In: Atkinson, B.G. (Ed.), Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells. Academic Press, San Diego, pp. 539–566.

Becker, K.B., Stephens, K.C., Davey, J.C., Schneider, M.J., Galton, V.A., 1997. The types 2 and 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. Endocrinology 138, 2989–2997.

Brown, D.D., Wang, Z., Furlow, J.D., Kanamori, A., Schwartzman, R.A., Remo, B.F., Pinder, A., 1996. The thyroid hormone-induced tail resorption program during *Xenopus laevis* metamorphosis. Proc. Natl. Acad. Sci. U.S.A. 93, 1924–1929.

Buckbinder, L., Brown, D.D., 1992. Thyroid hormone-induced gene expression changes in the developing frog limb. J. Biol. Chem. 267, 25786–25791.

Buscaglia, M., Leloup, J., deLuze, A., 1985. In: Bownes, M. (Ed.), Metamorphosis. Clarendon, New York, pp. 273–293.

Cooper, D.S., Kieffer, J.D., Saxe, V., Mover, H., Maloof, F., Ridgway, E.C., 1984. Methimazole pharmacology in the rat: studies using a newly developed radioimmunoassay for methimazole. Endocrinology 114, 786–793.

Crump, D., Werry, K., Veldhoen, N., Van Aggelen, G., Helbing, C.C., 2002. Exposure to the herbicide acetochlor alters thyroid hormone-dependent gene expression and metamorphosis in *Xenopus laevis*. Environ. Health Perspect. 110, 1199–1205.

Damjanovski, S., Amano, T., Li, Q., Ueda, S., Shi, Y.B., Ishizuya-Oka, A., 2000. Role of ECM remodeling in thyroid hormone-dependent apoptosis during anuran metamorphosis. Ann. N.Y. Acad. Sci. 926, 180–191.

Damstra, T., Barlow, S., Bergman, A., Kavlock, R., Van Der Kraak, G., 2002. Global Assessment of the State-of-the-Science of Endocrine Disruptors. World Health Organization, International Programme on Chemical Safety.

De Vito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A.O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McMaster, S., McCain, M., McConnell, E., Meier, C., Miller, R., Tietge, J., Tyl, R., 1999. Screening methods for thyroid hormone disruptors. Environ. Health Perspect. 107, 407–415.

Degitz, S.J., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Tietge, J.E., 2005. Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to model compounds 6-propylthiouracil, methimazole, and thyroxine. Toxicol. Sci. 87, 353–364.

Denver, R.J., Pavgi, S., Shi, Y.B., 1997. Thyroid hormone-dependent gene expression program for *Xenopus* neural development. J. Biol. Chem. 272, 8179–8188.

Frieden, E., 1968. Biochemistry of amphibian metamorphosis. In: Etkin, W., Gilbert, L.I. (Eds.), Metamorphosis: A Problem in Developmental Biology. Appleton-Century-Crofts, New York.

Helbing, C.C., Werry, K., Crump, D., Domanski, D., Veldhoen, N., Bailey, C.M., 2003. Expression profiles of novel thyroid hormone-responsive genes and proteins in the tail of *Xenopus laevis* tadpoles undergoing precocious metamorphosis. Mol. Endocrinol. 17, 1395–1409.

Hoopfer, E.D., Huang, L., Denver, R.J., 2002. Basic transcription element binding protein is a thyroid hormone-regulated transcription factor expressed during metamorphosis in *Xenopus laevis*. Dev. Growth Differ. 44, 365–381.

Huang, H., Marsh-Armstrong, N., Brown, D.D., 1999. Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that overexpress type III deiodinase. Proc. Natl. Acad. Sci. U.S.A. 96, 962–967.

Kawahara, A., Gohda, Y., Hikosaka, A., 1999. Role of type III iodothyronine 5-deiodinase gene expression in temporal regulation of *Xenopus* metamorphosis. Dev. Growth Differ. 41, 365–373.

Krain, L.P., Denver, R.J., 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. J. Endocrinol. 181, 91–104.

Leloup, J., Buscaglia, M., 1977. La triiodothyronine, hormone de la metamorphose des amphibiens. Comptes Rendues de l'Academie de les Sciences Paris Serie D 284, 2261–2263.

Nieuwkoop, P.D., Faber, J., 1956. Normal Table of *Xenopus laevis*. North Holland Publishing, Amsterdam.

Shi, Y.-B., 2000. Amphibian Metamorphosis: From Morphology to Molecular Biology. Wiley-Liss, New York.

Soldin, O.P., Braverman, L.E., Lamm, S.H., 2001. Perchlorate clinical pharmacology and human health: a review. Therap. Drug Monit. 23, 316–331.

Tietge, J.E., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Anderson, L.E., Wolf, D.C., Degitz, S.J., 2005. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. Environ. Toxicol. Chem. 24, 926–933.

Urbansky, E.T., 1998. Perchlorate chemistry: implications for analysis and remediation. Bioremediat. J. 2, 81–95.

Wang, Z., Brown, D.D., 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. J. Biol. Chem. 268, 16270–16278.

White, B.A., Nicoll, C.S., 1981. Hormonal control of amphibian metamorphosis. In: L.I.A.F., Gilbert, E. (Eds.), Metamorphosis: A Problem in Developmental Biology. Plenum Publishing, New York.

Wolff, J., 1998. Perchlorate and the thyroid gland. Pharmacol. Rev. 50, 89–105.